

The Effects of Rutin on the Gene Expression of *Dazl*, *Bcl2*, and *Caspase3* in Idarubicin-induced Testicular Damages in Mice

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Abstract

Background: Idarubicin (IDA), as a chemotherapeutic drug, has side effects on testicular tissue; different studies have shown the protective and antioxidant effects of rutin.

Objectives: The current study aimed at investigating the protective effects of rutin on damages induced by IDA in the testes of mice.

Methods: In the current experimental study, Balb/c mice were divided into 8 groups, including saline (10 mL/kg), rutin (single doses of 50 and 100 mg/kg via intraperitoneal (i.p) as the control groups, saline-IDA group (saline for 7 days, IDA, 10 mg/kg, i.p), rutin 50 and 100 mg/kg for 7 days before IDA, and rutin (single doses of 50 and 100 mg/kg) before IDA. The expression of *Bcl2*, *Caspase3* and *Dazl* at the mRNA level was assessed.

Results: Administration of rutin 100 mg/kg for 7 days before IDA could significantly downregulate *Caspase3* expression by 45% compared with saline-7d-IDA ($P < 0.001$). Also, in the R100-7d-IDA group, the expression level of *Dazl* (12.4 ± 3.50), *Bcl2* (2.5 ± 0.5) significantly increased compared to those of the saline-7d-IDA group (0.84 ± 0.5 , 0.12 ± 0.001 , respectively) ($P < 0.001$).

Conclusions: It seems that the preventive effects of rutin against damages caused by IDA can be attributed to its ability to reduce apoptosis, which may be mediated by underexpression of *Caspase3* and overexpression of *Bcl2* genes. Also, it could increase the expression of *Dazl* that may be important in spermatogenesis.

Keywords: Idarubicin, Rutin, Apoptosis, Spermatogenesis

1. Background

Fertility disorder is one of the main complications in patients with cancer, and infertility can be an important factor in the psychological morbidity of survivors (1). It is well known that chemotherapy induces azoospermia or reduces spermatogenesis (2). Idarubicin (IDA) is an effective drug for the early management of adult acute myeloid leukemia (AML), particularly in patients expressing high level of MDR (multiple drug resistant). IDA is a DNA intercalating agent, which reacts with topoisomerase II and has an inhibitory effect on nucleic acid synthesis (3). Doxorubicin and its derivative IDA, as anthracyclines, could prevent chromosomal segregation, and induce significant increases in the frequencies of disomic and diploid sperm (4). Epirubicin, another analogue of anthracyclines, could affect the abnormal reproductive outcomes by both the clastogenic and aneugenic potential in cancer survivors and medical personnel exposed to it (5). IDA could also induce oxidative stress, caspase activation, and apoptotic death in leukemic cell line (6). Therefore, reproductive toxicity induced by anthracyclines is a great concern for the

toxicities associated with chemotherapy and it is necessary to find a protective agent against it for patients. In this regard, the use of antioxidant agents can reduce the toxic effect of such drugs.

Rutin (3, 3', 4', 7 - pentahydroxyflavone - 3 - rhamnoglucoside) is a flavonoid of the flavonol-type found in many foodstuff and vegetables (7). Rutin has several pharmacological effects such as antioxidant properties (8, 9). It was shown that rutin (30 μ M) decreased lipid peroxidation induced by tert-butyl hydroperoxide in human sperm (10). Moreover, rutin as a flavonoid could restore motility of metal ($AlCl_3$, $CdCl_2$, and $PbCl_4$)-exposed sperm and protect it against lipid peroxidation (11). On the other hand, doxorubicin in some studies increased the expression of *Caspase3* (12, 13) and decreased *Bcl2* (13) in the testicular tissues of animals.

Furthermore, the DAZ (deleted in azoospermia) family refers to germ cell-specific transcription factors bound to RNA and play role in the regulation of several transcripts (14). It is expressed in primordial germ cells and/or pre-meiotic and meiotic germ cells of both genders, and the

most common cause of infertility is the deletion of DAZ gene in humans (15). Therefore, the current study aimed at assessing the protective effect of rutin by evaluating the expression of *Caspase3* as a proapoptotic and *Bcl2* as anti-apoptotic genes, and *Dazl* as a key gene involved in spermatogenesis in IDA-induced testicular damage in mice.

2. Methods

2.1. Animals

A total of 64 male Balb/c mice with weight range of 20 to 22 g were provided by Razi institute (Karaj, Iran) and maintained at a constant room temperature ($21 \pm 2^\circ\text{C}$) under a 12:12 light: dark cycle. All animals had free access to food and water. All experiments of the current study were conducted in accordance with the European communities council directive of 24 November 1986 (86/609/EEC).

2.2. Chemicals

IDA (4-demethoxydaunorubicin) was purchased from Pharmacia (Italia, S.P.A), rutin from Sigma (Sigma-Aldrich Co, Saint Louis, MO, USA), ketamine from Rotexmedica (GmbH, Germany), xylazine from Loughrea Co. (Galway, Ireland), RNeasy Mini Kit from Qiagene (Germany), and cDNA Synthesis Kit from Thermo Scientific, Fermentas (Waltham, MA, USA).

2.3. Experimental Design

In the current experimental study, mice were divided into 8 groups. The control group was given an intraperitoneal (i.p) injection of saline (10 mL/kg) daily for 7 days ($n = 8$). Two other groups, R50-7d and R100-7d, rutin (50 and 100 mg/kg, i.p) were given daily for 7 days (each group $n = 8$). In the saline-IDA group, saline (10 mL/kg, i.p) was given daily for 7 days, and on the last day, IDA (10 mg/kg, i.p) was injected 15 minutes after the administration of saline ($n = 8$). In the other 2 groups (R50-7d-IDA and R100-7d-IDA) rutin (50 and 100 mg/kg, i.p) was administered daily for 7 days, and on the last day, IDA (10 mg/kg, i.p) was injected 15 minutes after the administration of rutin (each group $n = 8$). In the 2 groups of R50-IDA and R100-IDA, rutin (50 and 100 mg/kg, i.p) was administered as a single dose, and on the same day, IDA (10 mg/kg, i.p) was injected 15 minutes after the administration of rutin (each group $n = 8$). Then, all of the mice in each group, 24 hours after treatment, were anaesthetized with i.p injection of ketamine (60 mg/kg)/xylazine (6 mg/kg) and were subsequently sacrificed. The testes of the animals were immediately removed, and cleaned with chilled 0.9% saline.

2.4. Expression Study of Candidate Genes

In the current study, testes tissues were homogenized using an ultrasonic processor UP100H (Hielsher, Germany), and then, RNAs were extracted from tissue samples using RNeasy Mini Kit (Qiagene, Germany). The quality and quantity of the isolated total RNA was measured using Nano Drop 2000c (Thermo, USA); thereafter, the RNA samples with A260/A280 ratios of > 2 were selected for the quantitative analysis. The extracted RNAs were, then, frozen at -80°C . First strand complementary DNA (cDNA) synthesis was also performed using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, Waltham, MA, USA). The *beta-actin* gene was used as an internal control to quantify target genes expression. Three target genes, such as *Dazl*, *Bcl2*, and *Caspase3*, and *beta-actin*, as internal control, were amplified with appropriate primers as follows:

Dazl F: GATGGACATGAGATCATTGGA, R: ATACCAGGGAG-CAATCCTGA

Bcl2 F: GAGTACCTGAACCGGCATCT, R: TTTGACCCA-GAATCCACTCA

Caspase3 F: TGTCATCTCGCTCTGGTACG, R: AAATGACCC-CTTCATCACCA

Beta-actin F: TTAGTGAGCTGCGTTTACAC, R: ACAAAGC-CATGCCAATGTTG.

Real-time polymerase chain reaction (PCR) was carried out in a total volume of 20 μL containing 10 μL Taq man master mix (Takara, Shiga, Japan), 0.2 μM forward and reverse primers, and 2 μL cDNA. Thermal cycling was performed on calibrated ABI-7500 (Applied Biosystems, Foster, CA, USA) sequence detection system using the following cycling condition: 30 seconds at 95°C as the first denaturation step, followed by 40 cycles at 95°C for 5 seconds and 60°C for 34 seconds. The $2^{-\Delta\text{CT}}$ method of relative quantification was used to determine the fold change in expression. It was performed by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control (*beta-actin*) in the treated and untreated samples ($\Delta\text{CT} = \text{CT}_{\text{target}} - \text{CT}_{\text{Beta}}$) (16).

2.5. Statistical Analysis

Statistical analysis, including mean and standard deviation (SD), was conducted using Prism (version 5) software. Additionally, one-way analysis of variance (ANOVA) and post hoc Tukey test were used to determine the significant differences between the studied groups. P value < 0.05 was considered as level of significance.

3. Results

According to the results of the current study, in saline-7d-IDA group, the expression of *Caspase3* as a proapoptotic

gene elevated significantly ($P < 0.001$). The administration of rutin 100 mg/kg for 7 days significantly downregulated *Caspase3* expression, compared with the saline-7d-IDA group ($P < 0.001$). However, administration of rutin 50 mg/kg for 7 days could not downregulate *Caspase3* expression and the difference between this group and saline was significant ($P < 0.05$). No significant difference was observed in the expression of *Caspase3* gene in R50 and R100-IDA groups, compared with the saline group (Table 1).

The expression of *Bcl2* gene, as an anti-apoptotic gene, between the saline-7d-IDA and saline groups was significant ($P < 0.01$). Overexpression of *Bcl2* gene in the mice receiving rutin 100 mg/kg for 7 days prior to IDA was shown compared with those of the saline-7d-IDA and saline groups ($P < 0.001$). However, similar to the saline-7d-IDA group, the expression of *Bcl2* gene in the treated group with rutin 50 mg/kg for 7 days before IDA was significantly downregulated, compared with the saline group ($P < 0.01$). Furthermore, the *Bcl2* expression significantly downregulated in the R50 and R100-IDA groups, compared with the saline group ($P < 0.01$) (Table 1).

The *Dazl* gene was significantly downregulated in the saline-7d-IDA group, compared with the saline group ($P < 0.05$). *Dazl* showed significant overexpression in the groups of R50 and 100-7d-IDA, compared with the saline-7d-IDA group ($P < 0.05$ and $P < 0.01$, respectively). There was no significant difference between R50 and R100-IDA groups, compared with the saline group (Table 1).

4. Discussion

In the current study, the expression of *Caspase3*, and *Bcl2* genes were over- and under-expressed, respectively in the saline-7d-IDA group. Therefore, IDA-induced oxidative stress in testicular cells leads to the induction of apoptosis in germ cells by causing the activation of *Caspase3* and downregulation of *Bcl2* gene. Similarly, the mRNA level of *Bcl2* decreased in spermatogonia, spermatocytes, and spermatids from testis tissues treated with 0.5 and 1 mM of doxorubicin (17). Underexpression and overexpression of *Caspase3*, and *Bcl2* genes were observed with rutin 100 mg/kg for 7 days in mice. Similarly, pretreatment of rutin could suppress the increment of Bax and *Caspase3*, and decrement of *Bcl2* expression induced by H_2O_2 at mRNA and protein levels (9). The protective effect of rutin on reproductive toxicity may be caused by scavenging reactive oxygen species (ROS), increasing superoxide dismutase, and catalase activities previously found in other studies (18-20). Generally, ROS produced as a result of oxidative stress, generate single strand break and double strand break in DNA by intrinsic mechanisms and causes adverse biological effects in cells. The biological consequences of exposure to

ROS include gene mutation, chromosome aberrations, cellular transformation, and cell death (21). Several studies showed that doxorubicin could induce apoptosis due to increase in the expression of various genes such as p38, p53, *Caspase3* and 9, and Bax/Bcl-xL, and downregulation of *Bcl2* (22, 23). Moreover, chronic administration of rutin (50 mg/kg, i.p, 3 w) protected testicular toxicity induced by adriamycin (doxorubicin) in rats. Rutin could increase testosterone and antioxidant enzyme levels, decrease testicular enzymes levels, and prevent expression of inflammatory markers (24).

In the current study, *Dazl* expression was evaluated in different studied groups. Rutin significantly upregulated *Dazl* expression dose dependently. Underexpression of *Dazl* gene in the saline-7d-IDA group was observed in the current study. Similarly, the microdeletion and sensitivity of DAZ gene (human homologue of *Dazl*) to the 4-Gy radiation were shown in other studies (25). The loss of *Dazl* expression results in apoptosis of the postmigratory germ cells (26).

In the current study, for the first time, the effect of rutin on the expression of *Caspase3*, *Bcl2*, and *Dazl* were studied in IDA-induced testicular damage in mice. Administration of rutin 100 mg/kg for 7 days increased the expression of *Bcl2* and *Dazl*, and decreased *Caspase3*. Furthermore, it seems that pretreatment and protection with 100 mg/kg of rutin for 7 days before IDA plays a more effective role when compared with a single dose of rutin. However, further studies are necessary on the sperm parameters, stress oxidative damage, and in the field of histopathology.

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Footnotes

Authors' Contribution: Mohammad Deihimi: performing the experiments and drafting the manuscript; Sahar Moghbelinejad: designing the study, performing molecular genetic assays, preparing and drafting the manuscript; Reza Najafipour: drafting the manuscript; Kazem Parivar: designing the study; Marjan Nassiri-Asl: designing the study, performing the statistical analysis, drafting and revising the manuscript. All authors read and approved the final manuscript. Mohammad Deihimi and Sahar Moghbelinejad contributed equally.

Table 1. Effect of Rutin on the Expression of *Caspase3*, *Bcl2*, and *Dazl* Genes in the Study Groups^a

Treatment	<i>Caspase3</i>	P Value	<i>Bcl2</i>	P Value	<i>Dazl</i>	P Value	Statistical Test
Saline	0.5 ± 0.15	-	0.95 ± 0.12	-	3.72 ± 0.99	-	Tukey-Kramer
R50-7d	0.45 ± 0.1	> 0.05	0.8 ± 0.35	> 0.05	2.07 ± 1.20	> 0.05	
R100-7d	0.4 ± 0.1	> 0.05	0.9 ± 0.3	> 0.05	3 ± 1.30	> 0.05	
Saline-7d-IDA	1.1 ± 0.25	< 0.001 ^b	0.12 ± 0.001	< 0.01 ^c	0.84 ± 0.5	< 0.05 ^d	
R50-7d-IDA	0.9 ± 0.3	< 0.05 ^d	0.45 ± 0.14	< 0.01 ^c	5.20 ± 1.20	> 0.01 ^c	
R100-7d-IDA	0.5 ± 0.23	< 0.001 ^e	2.5 ± 0.5	< 0.001 ^{b,e}	12.4 ± 3.50	< 0.001 ^{b,e}	
R50-IDA	0.55 ± 0.11	> 0.05	0.15 ± 0.08	< 0.01 ^c	1.05 ± .45	> 0.05	
R100-IDA	0.8 ± 0.12	> 0.05	0.23 ± 0.1	< 0.01 ^c	1.50 ± 0.63	> 0.05	

^aValues are expressed as mean ± SD.^bP < 0.001, compared to the saline group.^cP < 0.01, compared to the saline group^dP < 0.05, compared to the saline group^eP < 0.001, compared to the salins-7d-IDA group (n = 8), using Tukey-Kramer test.

Conflict of Interest: The authors declared no conflict of interest exists.

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